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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Wang et al.

Serial No.: 08/333,680

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For: NOVEL ADENOVIRAL VECTORS, Attorney Docket No.: 7639-061  
PACKAGING CELL LINES,  
RECOMBINANT ADENOVIRUSES,  
AND METHODS

#18  
chr

4-7-98

**DECLARATION OF QING WANG  
UNDER 37 C.F.R. §1.132**

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

I, Qing Wang, do declare that:

1. I am a scientist in the Molecular Biology Division of Cell Genesys, Inc., the assignee of the above-identified application. I have extensive experience in the field of molecular biology, particularly as it relates to adenoviral vectors and packaging cell lines, as evidenced by my curriculum vitae, attached hereto as Exhibit A.

2. The following analyses were carried out by me personally, or under my direction and supervision, to determine if an adenoviral packaging cell line, in particular a 293 embryonal kidney cell line, could be engineered to express the E4 early region gene and the E2A gene region, both under the control of an inducible promoter, in addition to the E1 early region gene, and if a such a cell line would survive and grow.

3. To test whether 293 cells can survive while expressing E1 and E4 early region genes and the cytotoxic E2A gene, I constructed the 293-ORF6/E2A cell line by introducing nucleotide sequences encoding the E4 early region gene in

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particular, the essential region open reading frame 6 (ORF6), under the control of the mouse  $\alpha$  inhibin promoter and nucleotide sequences encoding the E2A gene region under the control of the mouse  $\alpha$  inhibin promoter into 293 cells, and assayed for the presence of the E1 and E4 early region genes and the E2A gene region using the Southern blot assays described below. Southern blot analysis is a routinely used technique to identify DNA sequences from a mixture of DNA in particular genomic DNA, in order to detect the presence of a gene of interest. Detection of E1 and E4 early gene regions and E2A in genomic DNA isolated from 293-ORF6/2A cells indicates that these gene regions are present in the cells.

4. The following plasmids were used to introduce the E4-ORF6 early region gene and the E2A gene region into 293 cells: Plasmid pIK6.1MIP $\alpha$ -ORF6 comprises the minimum essential region of the E4-ORF6 early region gene, the ORF6 coding regions from nucleotide 1846 to 2756, as numbered from the right end of the Ad5 genome, linked to 238 base pairs of the inducible mouse  $\alpha$  inhibin promoter. Plasmid pIK6.1MIP $\alpha$ -E2A comprises the E2A gene region, from nucleotide 23435 to 24627, as numbered from the left end of the Ad5 genome, linked to 238 base pairs of the inducible mouse  $\alpha$  inhibin promoter.

5. The 293-ORF6/E2A cells were constructed as follows: 293 cells were grown as a monolayer in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose (JRH Biosciences, Denver, PA), 10% donor calf serum (Tissue Culture Biologies, Tulare, CA). Cells were seeded at  $5 \times 10^5$  per 10 cm plate. 10  $\mu$ g of pIK6.1 MIP $\alpha$ -ORF6 and 1  $\mu$ g of pGEM-pgkNeo.pghpoly plasmid encoding the neomycin resistant gene, were co-transfected into the 293 cells by calcium phosphate co-precipitation. The resulting G418 resistant clones were screened for the presence of the E4-ORF6 early gene region indicating a positive clone, designated 293-ORF6 cells. The 293-ORF6 cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM), 4.5 g/l glucose (JRH Biosciences,

Denver PA), 10% donor calf serum (Tissue Culture Biologies, Tulare, CA). Cells were seeded at  $5 \times 10^5$  per 10-cm<sup>2</sup> plate 24 hours prior to the transfection. Ten micrograms of pIK6.1MIP $\alpha$ -E2A plasmid (Figure 1) and 1 mg of pIK6.1 HYg.plasmid encoding the Hygromycin resistance gene under the control of the minimum CMV promoter, were co-transfected into 293-ORF6 cells by calcium phosphate co-precipitation. The hygromycin resistant clones were selected, screened by Southern blot analysis (Figure 2) and maintained in normal culture medium.

6. In order to determine the presence of E4-ORF6 and E2A in the transfected cells, eight cell lines were chosen for screening. Southern blot analysis of 293-ORF6/E2A cell lines was conducted as follows: 10  $\mu$ g of genomic DNA isolated from each cell line was digested with restriction enzymes Hind III and Dra III, electrophoresed on an 0.8% agarose gel and transferred to a nylon membrane. The nylon membrane, or blot, was first probed for E2A with a Bam H1-EcoRV fragment of the pIK6.1MIP $\alpha$ -E2A plasmid, and sequentially reprobbed for E4-ORF6 with a fragment of the ORF6 coding region (nucleotides 1846 to 2756), and E1 with a probe comprising a Hind III fragment of E1 early region gene (m.u. 7.7 to m.u. 17.1). The results indicate that the genomic DNA obtained from the 293-ORF6/E2A cell lines contain the coding region of E2A and E4-ORF6 gene regions in addition to the coding region of E1 (Figure 2).

7. The positively identified 293-ORF6/E2A cell lines have been grown in DMEM with 4.5 g/l glucose and 10% donor calf serum, and maintained in culture for at least twelve passages. These cell lines demonstrate similar growth rates and characteristics to those observed by the 293-ORF6 cells. These observations indicate that 293 cells transformed to express both the E4 early region gene and the E2A gene region, in addition to E1 early region gene, are able to survive and grow.

8. The data described in paragraph 6 demonstrates that a copy of the E1 early region gene, the E4-ORF6 gene region and the E2A coding region was successfully detected in the engineered 293-ORF6/E2A cell line indicating presence of the coding regions of E1 and E4 early region genes, in addition to the coding region of E2A. The observations described in paragraph 7 demonstrate that 293 cells transformed to express the E4 early region gene and the E2A gene region, in addition to the E1A early region gene, are able to survive and grow. The results and observations described above demonstrate that the methods taught in the above-identified patent application may be successfully applied to create a cell line expressing E1 and E4 early region genes and E2A under the control of an inducible promoter.

9. I declare further that all statements made in this Declaration of my own knowledge are true, and that all statements made on information and belief are believed to be true, and further, that these statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

2-2-98  
Date

  
Qing Wang